REMARKS

The Office Action dated April 3, 2003 presents the examination of claims 16-26 and 28-29. Claims 1-15 and 27 are withdrawn from consideration. Claims 17, 18, 23 and 27 are canceled herein. Claims 16, 19-22, 24-25, and 28-29 are amended. No new matter is inserted into the application.

Specification

The Examiner objects to the specification for various informalities. Applicants respectfully traverse.

(1) The Examiner asserts that the Brief Description of the Drawings for Figure 5 (page 10, lines 9-10 of the specification) does not indicate the difference between the black and white bars utilized in the Figure, nor the meaning of the numerical locations (1-5). Applicants amend the specification on page 10 in clarify that the black bars represent HGF administration group and the white bars represent Vehicle administration group, and that location 1 is the medial location of lower part of the thigh, location 2 is the medial location of the cnemial region, location 3 is the lateral location below knee, location 4 is the lateral location of anterior part of the cnemial region, location 5 is the lateral location in the lower part of the thigh, and location 6 is

the lateral location of the cnemial region. Support for this amendment to the specification is found in Example 2 of the specification, page 23, line 3 to page 24, line 24.

(2) The Examiner asserts that the Brief Description of the Drawings for Figure 7 (page 10, lines 21-23 of the specification) does not refer to Figures 7A-7C. Applicants amend the description to refer to Figures 7A-7C.

(3) The Examiner asserts that the difference between Figures 8 and 12 cannot be determined. Fig. 8 shows the result of determination of the I/N ratio (%) (blood pressure in an ischemic limb/blood pressure in a normal limb x 100) by intramuscularly administering HGF (10 μg/kg x twice) to a rat ASO model of hindlimb ischemia. The blood pressure in hindlimb was measured 14 days after removal of the femoral artery (see Example 5). Fig. 12 shows the result of determination of the I/N ratio by intramuscularly administering HGF (1 μg/kg x twice) to a rat ASO model of hindlimb ischemia. The blood pressure in hindlimb was measured 7 days after removal of the femoral artery (see Example 7).

(4) The Examiner objects to the title for allegedly not being descriptive. Applicants amend the title to --A METHOD OF TREATING ISCHEMIC DISEASE BY INTRAMUSCULAR ADMINISTRATION OF HEPATOCYTE GROWTH FACTOR--, as suggested by the Examiner.

Applicants respectfully submit that the objections to the instant specification are overcome. Withdrawal thereof is therefore respectfully requested.

Claim Objections

The Examiner objects to claims 16 and 23 for reciting a non-elected species. Claim 23 is canceled, thus rendering objection thereto moot. Applicants respectfully traverse the objection to claim 16. Claim 16 is amended to delete reference to the non-elected species of "arterial disease." Thus, the instant objection is overcome.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner rejects claims 16-26 and 28-29 under 35 U.S.C. § 112, first paragraph for allegedly containing subject matter not enabled by the specification. Claims 17, 18, and 23 are canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Specifically, the Examiner asserts that the specification enables a method for treating hindlimb ischemia in a patient in need

thereof, comprising: intramuscularly administering to a patient suffering from hindlimb ischemia an effective amount of a composition comprising HGF, wherein the composition is transported to, distributed in, and acts on tissues that are local to the region of administration, and wherein the composition has reduced transportation to, distribution in, and effect in blood or bodily organs other than those that are local to said region of administration. However, the Examiner asserts that the specification does not enable: (1) administration of HGF via a method other than intramuscular administration, (2) ischemia other than hindlimb ischemia, and (3) a method for preventing ischemic disease.

With regard to the Examiner's first assertion, Applications amend claim 16 to define the administration route as intramuscular. Support for intramuscular administration is found in the specification, such as on page 2, lines 21-24. With regard to the Examiner's third assertion, Applicants delete the term "preventing" from claim 16.

However, Applicants respectfully disagree with the Examiner's assertion that the instant specification fails to enable ischemia other than hindlimb ischemia. Applicants amend claim 16 to recite, "A method for treating ischemic disease of heart or extremities…." Support for this amendment is found in the specification, such as

on page 2, lines 21-24. As evidence of enablement, Applicants submit herewith two publications confirming that HGF can be administered intramuscularly to cardiac muscle in order to treat ischemic disease of the heart.

Specifically, Taketani et al., "Slow-Releasing of Hepatocyte Growth Factor via Minipellet Promise a Therapeutic Angiogenesis for Ischemic Heart Disease", *Circulation Journal*, 67(suppl. 1):632, PJ-590 (2003), shows that minipellets containing HGF embedded in cardiac muscle of rats having ischemic heart disease (i.e., myocardial infarction) released HGF and improved the cardiac function of the rats. See, Exhibit 1. Jayasankar et al., "Gene Transfer of Hepatocyte Growth Factor Attenuates Postinfarction Heart Failure", *Circulation*, 108(suppl. II):230-236 (2003), shows that direct intramyocardial injection of recombinant adenovirus encoding HGF into rats with myocardial infarction resulted in significantly improved myocardial function. See, Exhibit 2.

From these publications, it is clear that intramuscular administration of HGF in order to treat ischemic disease of the heart would not cause the skilled artisan undue experimentation. Thus, the subject matter of the claims is enabled by the specification. Withdrawal of the instant rejection is therefore respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner rejects claims 16-26 and 28-29 under 35 U.S.C. § 112, second paragraph for allegedly being indefinite. Claims 17, 18, and 23 are canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Specifically, the Examiner asserts that the claims do not have a step that clearly relates back to the preamble. In response to the Examiner's remarks, Applicants amend claim 16 to state that the ischemic disease of heart or extremities is treated. Thus, the instant rejection is overcome.

Rejection under 35 U.S.C. § 103(a)

The Examiner rejects claims 16-25 and 28-29 under 35 U.S.C. § 103(a), for allegedly being unpatentable over Goldberg '144 (U.S. Patent 6,498,144) in view of Pu et al. (*J Surg Res*, 54(6):575-583, (1993)). Claims 17, 18, and 23 are canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Goldberg '144 discloses a method for enhancing wound healing and a method of enhancing organ transplantation utilizing scatter factor (i.e., HGF). On page 8 of the Office Action, the Examiner states,

> *Goldberg et al. does not teach that HGF is transported to, distributed in, and acts on tissue local to the region of administration. Goldberg et al. also does not teach that HGF has reduced transportation to, distribution in, and effect in blood or bodily organs other than those that are local to the region of administration.*

The Examiner asserts that Pu et al. makes up for the deficiencies of Goldberg '144. Applicants respectfully disagree. Pu et al. fails to teach or suggest the deficiencies of Goldberg '144, as noted below.

As explained on page 3, line 25 to page 7, line 25 of the instant specification, the present invention was devised on the basis of the following findings: (1) intramuscular administration of HGF maintains the HGF at a very high concentration at the site of administration, (2) unlike systemic administration such as in serum, liver, and kidneys, transportation or action in regions other than the region of illness (site of administration) is very small, and (3) a substantial effect is obtained at low dose.

In other words, the present invention is devised on the basis of the findings that the intramuscular administration of HGF in the

ischemic region is an unconventionally effective and safe method for the treatment of ischemic disease.

On the other hand, Pu et al. experimented in the following five groups only:

Group 1: Intramuscular administration of 4 mg ECGF in ischemic region (Fig. 1A);

Group 2: Intramuscular administration of 1 mg ECGF in ischemic region (Fig. ,A);

Group 3: Administration of saline;

Group 4: Intramuscular administration of 4 mg ECGF in forelimb apart from ischemic region (Fig. 1B);

Group 5: Intramuscular administration of 4 mg ECGF in normal rabbits (Fig. 1C).

Although Pu et al. notes that administration of growth factors locally rather systemically results in a more beneficial effect, Pu et al. suggests nothing about the superior effectiveness of local administration as compared with systemic administration. In other words, Pu et al. fails to teach or disclose that systemic administration is inefficient compared to direct administration. Thus, Pu et al. teaches nothing about the features of the present invention, that is, HGF by intramuscular administration is maintained at very high concentration at the site of

administration, with very small transportation or action in other regions than the region of illness, such as serum, liver, and kidneys.

With regard to dosage, according to Pu et al., "... the negative results from this study might relate to factors such as the short half-life and comparably small dosage of the agent." That is, multiple or continuous direct administrations of angiogenic growth factor using higher dosage may be necessary to produce an angiogenic effect in the *in vivo* setting (see, page 582, left column, lines 24-29). This feature of Pu et al. is a reverse concept of the "low dosage" of the present invention and therefore teaches away from the present invention. Indeed, Pu et al. administered 4 mg of ECGF for 10 days (a total of 40 mg), whereas the present invention produced a substantial effect at a very low dose of HGF 100 μg/kg x 2 times (a total of 0.6 mg) in Example 6 (using the same type of rabbits as in Pu et al.).

Thus, Pu et al. does not clarify the therapeutic effect as compared with the conventional systemic administration, or action on regions other than the site of administration, and, in actuality, recommends increasing dosage, and further uses ECGF which is a different factor from HGF used in the present invention. For these reasons, Pu et al. fails to make up for the deficiencies

of Goldberg '144.For these reasons, Pu et al. fails to make up for the deficiencies of Goldberg '144.

In conclusion, therefore, the cited references fail to render the present invention obvious. Withdrawal of the instant rejection is therefore respectfully requested.

Conclusion

Applicants respectfully submit that all outstanding rejections/objections of record have been properly accommodated such that the present application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee of $950.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any

overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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2520-0120P

Attachments: Exhibit 1: Taketani et al.
Exhibit 2: Jayasankar et al.

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ABSTRACTS

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AM and α-CGRP were significantly attenuated by α-CGRP-(8-37), but not by AM-(22-52). By contrast, cells transfected with CRLR/RAMP2 responded more selectively to AM; in this case, the effect was blocked by AM-(22-52), but not by α-CGRP-(8-37). In HCASMC, α-CGRP-(8-37) significantly blocked α-CGRP- and AM-induced increases in cAMP, whereas AM-(22-52) did only the responses induced by AM.Taken together, these results suggest that CRLR/RAMP1 fully functions as an AM receptor as well as a CGRP receptor, which may explain why many of the actions of AM are potently antagonized by α-CGRP-(8-37).

PJ-588

Crosstalk between c-Src- and Smad-Dependent Pathways Regulates the TGF-β-Induced SMC-Specific Gene Expression in Murine Embryonic Fibroblast 10T1/2 Cells

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TGF-β is implicated in the control of the SMC differentiation. Previous study has described that TGF-β increases the expression of the SMC-specific genes in murine embryonic fibroblast 10T1/2 cells. In this study we investigated the molecular mechanisms by which TGF-β increases the SM22α gene. We showed that an increase in SM22α was inhibited by PP1, an inhibitor of Src family kinase. Western blot anaysis showed that TGF-β rapidly induced phosphorylation of c-Src. This response was completely inhibited by PP1, suggesting that c-Src is a major tyrosine kinase in TGF-β signaling pathway. To confirm these results, we employed Src++ cells (c-Src++, Yes-, Fyn-) and SYF cells (c-Src-, Yes-, Fyn-). Induction of the SM22α gene was markedly attenuated in SYF cells as compared with Src++ cells. PP1 also inhibited the TGF-β-induced expression of serum response factor (SRF), a transcription factor, which directly regulates the SM22α gene expression, suggesting that activation of c-Src by TGF-β induces SRF expression. Moreover, we showed that Smad6, an inhibitory Smad, abolished the TGF-β-induced SM22α induction, suggesting that Smad-dependent pathway also regulates the TGF-β-induced SM22α gene expression. These results provide the first evidence that TGF-β activates the Src tyrosine kinase which leads to the activation of Smad- and SRF-dependent transcription of the SM22α gene, and imply that crosstalk between c-Src- and Smad-pathways is required for TGF-β-induced SMC-specific genes.

PJ-589

Control-Released Hepatocyte Growth Factor (HGF) Prevents Myocardial Fibrosis in Spontaneously Hypertensive Rats

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Introduction: We have developed HGF-incorporating gelatin hydrogel sheet which was designed to release HGF over 2 weeks. We hypothesized that HGF sheet would prevent the progression of cardiac fibrosis and heart failure in spontaneously hypertensive rats (SHRs). **Methods:** SHRs were randomized into two groups: HGF groups (n=9) and control group (n=9). In the HGF group, HGF sheet was placed on the epicardial surface of the LV free wall through a small pericardiotomy. Doppler- echocardiographic study and measurements of collagen fraction and tissue levels of endotherin-1 (ET-1) were performed 4 weeks after the surgery. **Results:** There were two deaths in the control group. LV end-diastolic dimension in the HGF group was significantly smaller than that in the control group (HGF, control; 7.2±0.2, 8.7 ±0.3mm, respectively, $P<0.01$). Fractional shortening in the HGF group was significantly higher than that in the control group (51±3, 39±3%, $P<0.05$). The rate of E-wave deceleration in the HGF group was significantly lower than that in the control group (26.38±1.34, 35.16±2.70 m/sec^2, $P<0.01$). E/A-wave velocity ratio in the HGF group was significantly lower than that in the control group (3.9±0.2, 5.9±0.4, $P<0.01$). HGF significantly attenuated the collagen fraction and tissue ET-1. **Conclusions:** Control-released HGF prevented the progression of cardiac fibrosis and improved LV systolic and diastolic function in SHRs.

PJ-590

Slow-Releasing of Hepatocyte Growth Factor via Minipellet Promise a Therapeutic Angiogenesis for Ischemic Heart Disease

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Therapeutic angiogenesis is a promising strategy for patients with ischemic heart disease. However, gene therapy has still limitations such as safety and ethical problems. And more safe optimal method of therapeutic angiogenesis is desired. Minipellet is a useful method to ensure the slow-release of protein. In this study, we proved that slow release of Hepatocyte growth factor (HGF), a potent angiogenic factor, using minipellet may promise a therapeutic angiogenesis for ischemic heart disease. We prepared the HGF minipellets including 200 μg of HGF in 5 mm length and 0.5 mm diameter. Two minipellets were inserted to infarct rat heart 4 weeks after LAD ligation (P group, n=5). Saline (C group, n=6) or HVJ liposome bearing HGF cDNA (G group, n=7) were injected. HGF were released from the minipellet for 4 consecutive weeks. In the P and G group, cardiac remodeling was significantly attenuated and diastolic function was significantly improved compared with the C group. Capillary density in the treated myocardium was significantly higher in the P and the G group. Moreover, immunohistochemical analysis revealed that HGF released from minipellets were distributed to whole heart in the P group. In conclusion, slow release of HGF using minipellet promise a same potency of therapeutic angiogenesis as gene therapy.

Stroke/ Cerebrovascular Circulation (H)

PJ97

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PJ-591

Magnetic Resonance Imaging Analysis Demonstrates Efficacy of Recombinant Annexin II in a Rat Model of Stroke

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Background and purpose: Annexin II is identified as a co-receptor on endothelial cell surface for plasminogen and tissue plasmin activator. In this study, we examined the effect of recombinant annexin II (rANII) in a rat model of embolic stroke. **Methods:** The right external carotid artery of male Wistar rats was cannulated. Autologous clots were infused immediately after injection of rANII (1mg/kg) or saline (1mg/kg) to develop emboli in the area of the right middle cerebral artery. Apparent diffusion coefficient (ADC) and relative blood flow (rCBF) were measured by using magnetic resonance imaging (MRI). **Results:** Three hours after embolization, the tissue volume with low ADC values (an MRI index of tissue damage) was significantly decreased in rANII-treated group (9.62+/-2.14% of ipsilateral hemisphere) compared to saline-treated group (16.67+/-3.91%). Serial rCBF measurement revealed significant improvement of blood flow in the rANII-treated group (73.54+/-11.21% of contralateral hemisphere at 3 hrs) compared to saline treated group. (59.25+/-10.17%) The infarcted area in the H&E stained section from both groups matches the ADC declined area in MRI. Moreover, Kruber Barrera stained section revealed a dramatic reduction of neuronal cell death in the periinfarcted region of rANII-treated group. **Conclusion:** Consecutive

Gene Transfer of Hepatocyte Growth Factor Attenuates Postinfarction Heart Failure

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Background—Despite advances in surgical and percutaneous coronary revascularization, ongoing ischemia that is not amenable to standard revascularization techniques is a major cause of morbidity and mortality. Hepatocyte Growth Factor (HGF) has potent angiogenic and anti-apoptotic activities, and this study evaluated the functional and biochemical effects of HGF gene transfer in a rat model of postinfarction heart failure.

Methods and Results—Lewis rats underwent ligation of the left anterior descending coronary artery with direct intramyocardial injection of replication-deficient recombinant adenovirus encoding HGF (n=10) or empty null virus as control (n=9), and animals were analyzed after six weeks. Pressure-volume conductance catheter measurements demonstrated significantly preserved contractile function in the HGF group compared with Null control animals as measured by maximum developed LV pressure (79±5 versus 56±4 mm Hg, $P<0.001$) and maximum dP/dt (2890±326 versus 1622±159 mm Hg/sec, $P<0.01$). Significant preservation of LV geometry was associated with HGF treatment (LV Diameter HGF 13.1±0.54 versus Null 14.4±0.15 mm $P<0.01$; LV wall thickness 1.73±0.10 versus 1.28±0.07 mm $P<0.01$). Angiogenesis was significantly enhanced in HGF treated animals as measured by both Von Willebrand's Factor immunohistochemical staining and a microsphere assay. TUNEL analysis revealed a significant reduction in apoptosis in the HGF group (3.42±0.83% versus 8.36±1.16%, $P<0.01$), which correlated with increased Bcl-2 and Bcl-x_L expression in the HGF animals.

Conclusions—Hepatocyte Growth Factor gene transfer following a large myocardial infarction results in significantly preserved myocardial function and geometry, and is associated with significant angiogenesis and a reduction in apoptosis. This therapy may be useful as an adjunct or alternative to standard revascularization techniques in patients with ischemic heart failure. **(*Circulation*. 2003;108[suppl II]:II-230-II-236.)**

Key Words: angiogenesis ■ heart failure ■ gene therapy ■ apoptosis

Ischemic cardiac disease that is not amenable to conventional revascularization poses a significant therapeutic challenge. Despite advances in conventional surgical and percutaneous revascularization techniques, more than 10% of patients referred to tertiary intervention centers have symptomatic coronary artery disease (CAD) that cannot be revascularized.[1] Ischemia induces endogenous myocardial angiogenesis, but the result of this process often does not adequately compensate.[2] The exogenous induction of angiogenesis in ischemic hearts may provide improved perfusion to support the remaining myocardium. Numerous growth factors have been utilized in attempts to induce therapeutic angiogenesis by gene transfer or as recombinant proteins, with mixed results in clinical trials to date.[3–5]

Hepatocyte Growth Factor (HGF) is a heterodimeric pluripotent growth factor with an 80-kDa apparent molecular weight that has been shown to have potent angiogenic actions on various cells types.[6] In addition, HGF has anti-apoptotic effects,[7] blocking the programmed cell death response that is known to contribute significantly to the development of ischemic heart failure.[8]

Because of its pluripotent effects overexpression of HGF may be particularly effective in treating post-ischemic heart failure. This study was designed to investigate the functional, angiogenic and anti-apoptotic effects of adenoviral-mediated gene transfer of HGF in a rat model of postinfarction heart failure.

Materials and Methods

Animal Care

All animals received humane care in compliance with the "Guide for the Care And Use of Laboratory Animals," Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, D.C., 1996). The study was conducted in accordance with the animal care and use

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guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

Adenoviral Vector Construction

Replication-deficient (E1, E3 deleted) adenoviral vectors containing the rat HGF transgene driven by the human muscle creatine kinase (MCK) promoter were made and obtained from the Institute of Human Gene Therapy, University of Pennsylvania (Adeno-HGF). The MCK promoter was chosen to provide high levels of striated muscle-specific expression. Empty replication-deficient adenovirus containing no transgene was similarly obtained as a control (Adeno-Null).

Animal Surgery

Twenty-nine male Lewis inbred rats (250 to 300 g, Charles River Laboratories) were used for main portion of this study. Lewis rats were chosen due to their consistent infarct size of approximately 35% of the left ventricle and low mortality after ligation of the left anterior descending coronary artery (LAD).[9]

Rats were anesthetized with intraperitoneal doses of ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated with 0.5% isoflurane at the time of surgery. A left thoracotomy was performed through the fourth interspace, the pericardium was opened, and the proximal LAD artery was encircled with a 7-0 polypropylene suture. Animals were then randomized to 1 of 3 experimental groups: Sham, Adeno-Null (Null), or Adeno-HGF (HGF). In the Sham group (n=10) the suture was removed without tying. In the Null (n=9) and HGF (n=10) groups the suture was tied to create a large anterior left ventricular infarct. Infarction was confirmed by visible blanching of the region at the time of ligation. All animals in the Null group then received direct intramyocardial injections of 5×10^9 pfu of Adeno-Null virus into the infarction border zone area via a 30 gauge needle. A total volume of 250 μL was injected into 5 separate areas. Animals in the HGF group received similar injections of Adeno-HGF into the border zone. The animals were then closed in three layers and recovered for 6 weeks.

Hemodynamic Measurements

After six weeks the animals were once again anesthetized, intubated, and mechanically ventilated, and a repeat thoracotomy was performed. A 2 French pressure-volume conductance catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle through the apex of the heart. Hemodynamic measurements were recorded and analyzed using the ARIA™ 1 Pressure Volume Analysis software (Millar Instruments, Houston, TX). The heart was then arrested in diastole by injection of 0.1 cc of KCl (1 mEq/mL), and the left ventricular cavity was filled with OCT embedding compound fixative retrograde through the transected aortic root. The catheter was removed and the aortic root was ligated. The distended heart was then placed in a container of OCT embedding compound, bathed in isopentane, frozen in liquid nitrogen, and stored in a −80°C freezer.

Ventricular Geometry

In all groups, four adjacent sections midway between the base and apex spanning the border zone and perpendicular to the longitudinal axis of the ventricle were obtained. Measurements were performed on digitized photomicrographs using Openlab image processing software (Improvision, Lexington, MA) with standards of known length, and were obtained on 2 representative sections for each animal. For chamber size, left ventricular diameter was recorded in both vertical and horizontal axes and averaged. For border-zone wall thickness, measurements were obtained on two separate areas for each section and averaged. A single investigator blinded to the treatment groups performed all measurements. The results are reported for each group as the average chamber diameter and wall thickness in mm±SEM.

Assessment of Angiogenesis by Immunohistochemistry and Microsphere Assay

For immunohistochemistry, frozen heart specimens were obtained from storage in a −80°C freezer warmed to approximately −22°C in the cryostat. Four 10 μm sections were then prepared with a cryostat from the point of LAD ligation to the apex of the heart at 0.25 cm intervals. The sections were subsequently blocked with 5% BSA in PBS, and immunohistochemical staining was performed using a mouse monoclonal antibody to the endothelial cell marker von Willebrand's Factor (Cedar Lane Laboratories, Hornsby, ON) at a dilution of 1:50. An alkaline phosphatase conjugated secondary antibody was used at a dilution of 1:500. The 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) Liquid Substrate System (Sigma, St. Louis, MO) was used for detection. The specimens were then counterstained with eosin, and quantitative assessment of endothelial cells (ECs) was obtained through four representative border zone high-powered fields (hpf) by a single investigator blinded to the treatment group. The results are reported as the number of endothelial cells per hpf±SEM.

To further quantify the extent of induced angiogenesis, an additional 18 animals were utilized for a microsphere assay (Null n=9, HGF n=9). Animals underwent LAD ligation and virus delivery as described above. Six weeks later the rats underwent repeat thoracotomy and, instead of hemodynamic measurements, the slow injection of 1.5×10^5 microspheres of 15 μm diameter (Triton Technology, San Diego, CA) was performed directly into the left ventricular cavity. The heart was allowed to beat for approximately 1 minute before treatment with KCl as described above. Biopsy specimens were obtained from the entire borderzone area of the left ventricle and snap frozen in liquid nitrogen. Specimens were removed from liquid nitrogen and processed according to the manufacturer's specifications. The number of microspheres/gram of tissue was calculated based on spectrophotometric absorbance measurements and comparisons to known standards supplied by the manufacturer. As the spheres lodge in the capillary circulation, the number of spheres obtained is presumed to be proportional to the functional capillary density of the tissue.

TUNEL Assay

Frozen heart specimens were sectioned as described above, the TUNEL assay was performed with a TdT-FragEL™ DNA Fragmentation Detection Kit (Oncogene Research Products, Boston, MA), and tissue slides were subsequently incubated with terminal deoxynucleotidyl transferase biotin-labeled dUTP. Streptavidin-horseradish peroxidase conjugate binds to biotin to produce an insoluble colored precipitate with diaminobenzidine. Counterstaining with methyl green was performed to visualize normal nuclei. The apoptotic index was calculated as: (number of apoptotic nuclei/number of total nuclei) * 100. Four measurements were obtained from the border zone area of each section by a single investigator blinded to the treatment group. The results are reported for each experimental group as the average apoptotic index±SEM.

Western Blotting

Ventricular biopsy specimens obtained for Western blotting were snap-frozen in liquid nitrogen without OCT fixative. Specimens were sheared with a 25 gauge needle after homogenization in 10 volumes of SDS lysis buffer (100 Mm Tris, pH 8.0, 10% SDS, 10 mmol/L EDTA, 50 mmol/L DTT). After normalizing for protein content, 50 μg of each sample were electrophoresed on a 12.5% SDS-polyacrylamide gel following 10 minutes of denaturation at 100°C. A wet transfer apparatus was then used to transfer proteins to Immobilon-P membranes (Millipore, Bedford, MA), which were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Immunoblotting was performed using the polyclonal antibodies against HGF (1:200 dilution) (NeoMarkers, Fremont, CA), Bcl-x_L (1:500), and Bcl-2 (1:500) (both from Santa Cruz Biotechnology, Santa Cruz, CA). The ECL kit (Amersham, Piscataway, NJ) was used for detection and relative expression levels were quantified on a Macintosh computer using the public

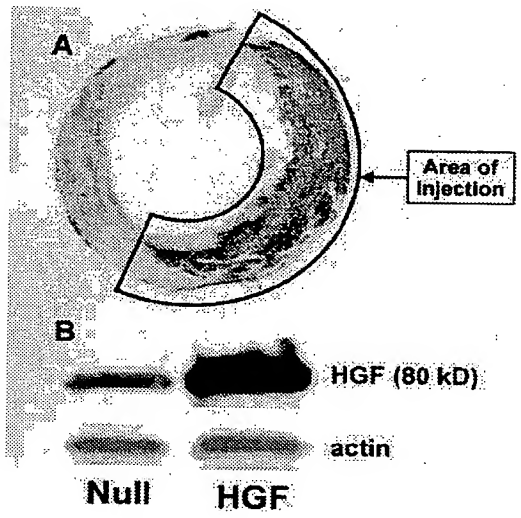

Figure 1. (A) Representative cross-section of a rat heart one week following direct injection of an adenoviral vector containing the beta-galactosidase gene. X-gal staining reveals expression throughout the area of injection in the left ventricular free wall. (B) Immunoblotting for the hepatocyte growth factor protein 6 weeks following infarction and virus delivery, with actin staining demonstrating equivalent protein loading between lanes. Null=Adeno-Null virus delivery, HGF=Adeno-HGF virus delivery.

domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.gov/nih-image).

Statistical Analysis

All values are expressed as mean±SEM. The unpaired Student's t-test was used to calculate the statistical significance between the means of 2 groups. Comparisons between more than 2 groups were analyzed by ANOVA followed by Tukey-Kramer post hoc testing. A probability value of less than 0.05 was considered to be significant.

Results

Viral Delivery and Transgene Expression

Virus delivery and transgene expression using our method of direct intramyocardial injection was confirmed using a replication-deficient E1, E3-deleted adenovirus containing the beta-galactosidase gene under control of the cytomegalovirus promoter. The virus was delivered by direct injection to a rat heart as described above. The rat was sacrificed after 1 week and X-gal staining was performed (Figure 1). Transmyocardial expression was observed in over 85% of the left ventricular free wall area of injection. Immunoblotting for HGF protein expression performed on myocardial biopsy specimens in Null and HGF treated animals showed significant overexpression in the HGF treated group 6 weeks after injection (Figure 1).

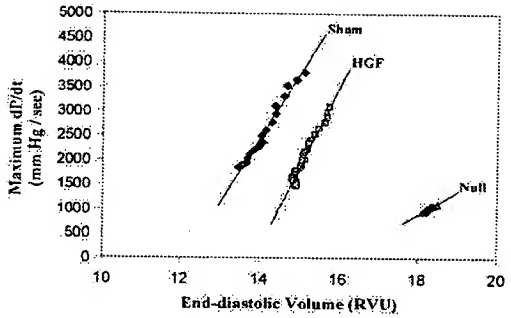

Figure 2. Representative cardiac contractility curves for a single animal in each experimental group as measured by a pressure-volume conductance catheter at the time of sacrifice. Contractility is measure as the slope of maximum dP/dt versus end-diastolic volume. Sham=sham thoracotomy, Null-Adeno-Null group, HGF=Adeno-HGF group, RVU=relative volume unit.

Hemodynamics and Cardiac Function

The Adeno-HGF group had significant preservation of cardiac contractile function as measured by maximum developed left ventricular pressure and maximum dP/dt compared with the Adeno-Null control group (Table 1). A sensitive measure of the contractile state of the heart is the slope of the maximum dP/dt versus end-diastolic function curve, and significant preservation of contractile function was observed in the HGF group compared with the Null controls (Figure 2). There was no significant difference in cardiac function between sham, uninfarcted animals, and infarcted animals treated with Adeno-HGF.

Ventricular Geometry

The Adeno-HGF group demonstrated significant preservation of normal left ventricular geometry compared with the Adeno-Null controls (Figure 3). The HGF treated animals had reduced chamber dilatation compared with Null controls (HGF 13.1±0.54 versus Null 14.4±0.15 mm, $P<0.01$; Sham 12.2±0.17 mm). The HGF group also demonstrated significantly less border zone wall thinning when compared with the Null animals (HGF 1.73±0.10 versus Null 1.28±0.07 mm, $P<0.01$; Sham 1.70±0.11 mm).

Cardiac Function Parameters

Group	n	Heart Rate (bpm)	Maximum LV Pressure (mm Hg)	Maximum LV dP/dt (mm Hg/sec)
Sham	10	235±12	82.8±2.6	3123±125
Null	9	253±9	56.0±3.9	1622±159
HGF	10	257±10	79.4±5.0	2890±326
p (HGF versus Null)		NS	<0.001	<0.01

Data were obtained 6 weeks after initial surgery and are presented as mean±SEM.

LV, left ventricular; Sham, sham thoracotomy group; Null, Adeno-Null injected group; HGF, Adeno-HGF injected group; NS, not significant ($P>0.05$); statistical analysis by ANOVA with Tukey-Kramer post-hoc testing.

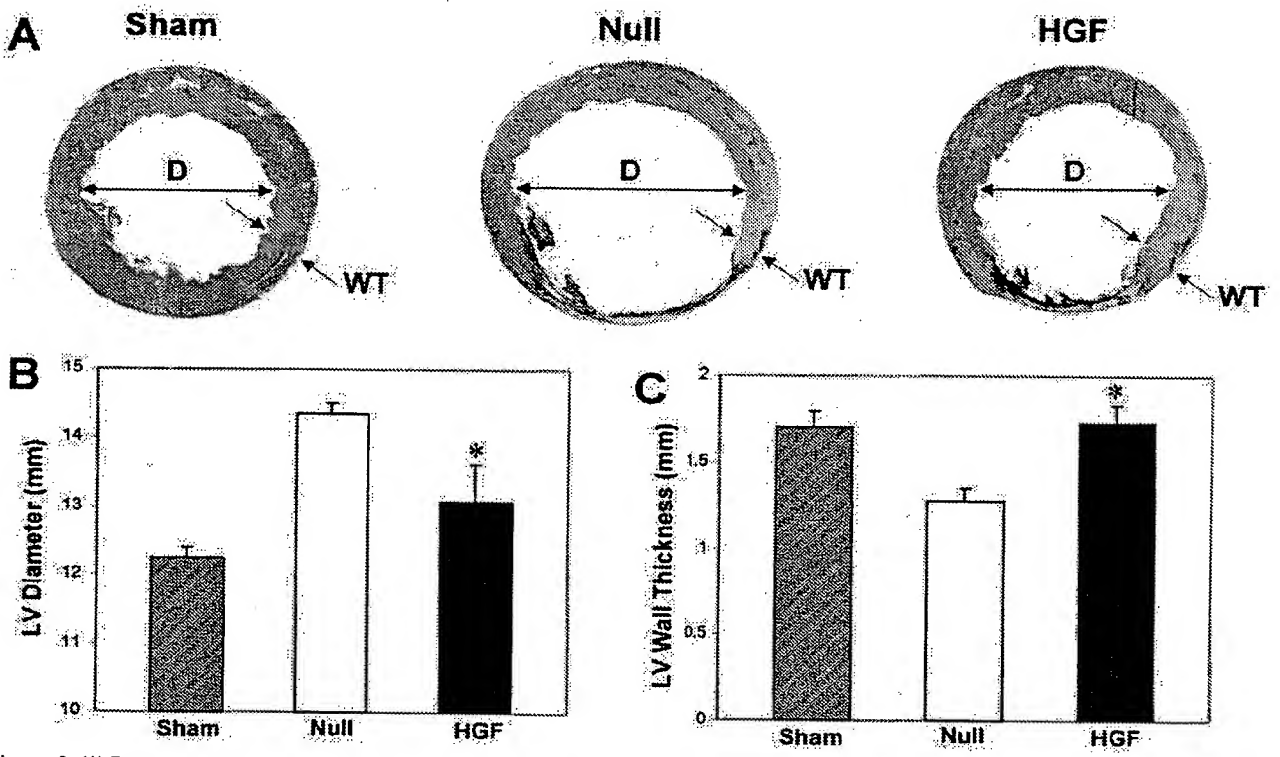

Figure 3. (A) Representative cross-sections of a single rat heart from each experimental group 6 weeks following initial surgery. Hematoxylin and eosin staining has been performed, and arrows depict the diameter (D) and wall thickness (WT) measurement areas. (B) Graph of average left ventricular chamber diameter in each experimental group at the study end point. (C) Graph of average left ventricular free wall thickness in each experimental group at the conclusion of the study (Sham n=10, Null n=9, HGF n=10). *P<0.01 versus Null control.

Angiogenesis: VWF Immunohistochemistry and Microsphere Assay

Immunohistochemical staining for von Willebrand's Factor revealed an approximately two-fold increase in endothelial cells per high-powered field in the Adeno-HGF group compared with both Null and Sham control groups (Figure 4). The HGF group had 169±11 ECs/hpf compared with 95±7 in the Null (P<0.001), and 103±16 in the Sham controls. The functional capillary density, as calculated by the microsphere assay, was over 50% greater in the Adeno-HGF group compared with Null control animals (13 624±1436 versus 8829±1477 microsphere/gram tissue, P<0.05) (Figure 5).

TUNEL Analysis

TUNEL assay analysis revealed that the HGF treated animals had a >50% reduction in the percentage of apoptotic cells in the border zone area compared with Null control animals (3.42±0.83% versus 8.36±1.16%, P<0.01) (Figure 6). Sham uninfarcted animals had an apoptosis rate of 0.47±0.07%.

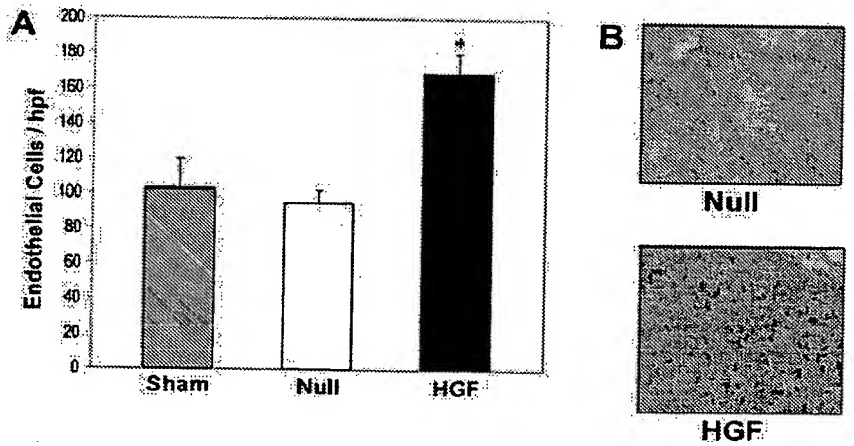

Figure 4. A, The average number of endothelial cells per high-powered field (hpf) in the border-zone for each experimental group as determined by von Willebrand's Factor (VWF) immunohistochemical staining. B, Representative VWF-stained high-powered fields. Endothelial cells are stained dark purple against a pink background (original magnification ×200). *P<0.001 versus Null control.

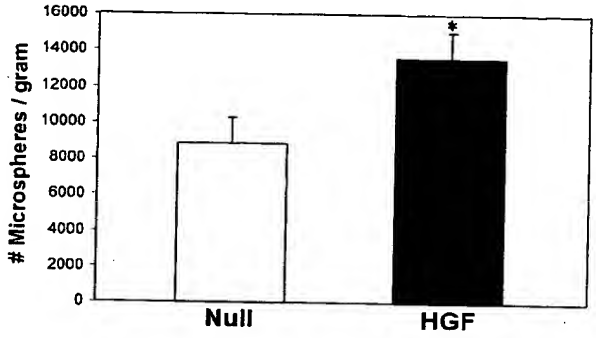

Figure 5. The average number of microspheres per gram of left ventricular border zone tissue for the Null and HGF experimental groups at the study end point. Microspheres were injected at the time of sacrifice and were quantified using spectrophotometric absorbance. N=9 in each group, *$P<0.05$ versus Null control.

Bcl-2 and Bcl-x_L Expression

Immunoblotting performed against Bcl-2 and Bcl-x_L revealed significant overexpression of both proteins in HGF treated animals (Figure 7). The relative expression of Bcl-2 was 3.92 ± 0.54 in the HGF group compared with 1.04 ± 0.25 in the Null group (arbitrary units, $P<0.001$). The relative expression of Bcl-x_L was 3.44 ± 0.56 in the HGF group compared with 1.88 ± 0.27 in the Null group ($P<0.01$). Interestingly Bcl-x_L, but not Bcl-2, was elevated in infarcted control animals that received null virus injections. All expression quantification was normalized to the Sham animals, which had relative expression levels of 1.0 for both proteins.

Discussion

This study demonstrates that adenoviral-mediated gene transfer of HGF results in significant preservation of contractile function 6 weeks following a large myocardial infarction in rats. In addition, more favorable geometry was associated with Adeno-HGF administration, with decreased left ventricular dilatation and preserved wall thickness. HGF-treated animals had significant angiogenesis as well as a reduction in apoptosis levels. Importantly, continued overexpression of the HGF protein was demonstrated at the 6-week time point.

Hepatocyte Growth Factor is both a potent angiogenic and anti-apoptotic agent.[6,7] Previous studies have demonstrated increased levels of the HGF receptor, encoded by the proto-oncogene c-Met, following a large myocardial infarction[10] and in response to hypoxia in animal models of myocardial ischemia.[11] HGF itself may upregulate the c-Met receptor and thus activate an autocrine feedback loop,[12] and blocking endogenous HGF has been shown to worsen cardiac failure and increase mortality in a rat model of ischemic injury.[13] The c-Met receptor is present on both endothelial and vascular smooth muscle cells, and angiogenesis may be stimulated directly and/or indirectly via the induction of VEGF secretion by smooth muscle cells.[14] The post-infarction and ischemic milieu, therefore, provides particularly favorable conditions for HGF to exert its effects via upregulation of the c-Met receptor.

Recently it has been shown that serum levels of HGF are elevated following myocardial infarction and ischemia in human patients.[15] In a study by Watanabe and colleagues in which serum and coronary sinus HGF levels were measured, decreased local HGF production with increased extraction of serum HGF by the heart was seen in patients with CAD versus normal controls.[16] It has also been observed that enhanced secretion of HGF from the infarct zone is associated with decreased ventricular dilatation and improved perfusion following myocardial infarction in human patients.[17] Taken together, these results suggest that while serum HGF levels are increased under conditions of cardiac ischemia, local production of HGF by ischemic and infarcted myocardium is decreased. Increased serum HGF may be a protective response to limit continued poor perfusion following an ischemic insult. In contrast, downregulation of local HGF production appears to be a maladaptive response. Local overexpression of HGF appears to correlate with more favorable geometry and perfusion, making it an ideal therapeutic target.

We propose that the preservation of cardiac function observed in this study is due to the dual angiogenic and anti-apoptotic activities of HGF. Angiogenesis in the border zone may rescue a significant fraction of cardiomyocytes that would otherwise be lost or non-functional due to ongoing

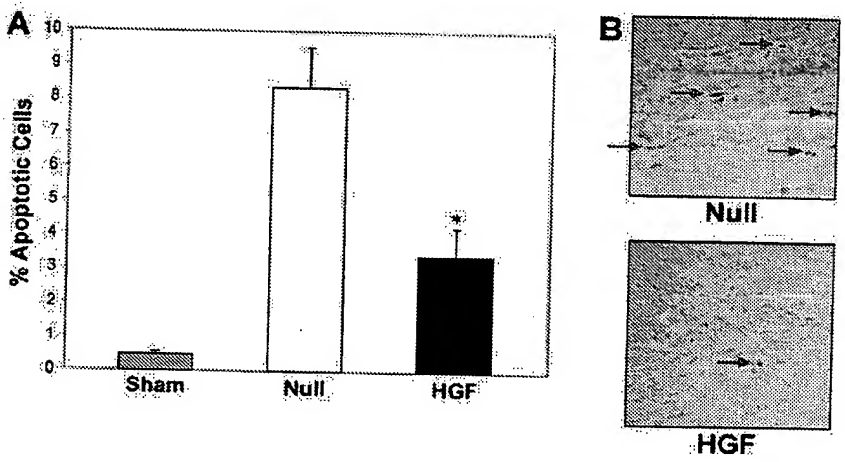

Figure 6. (A) Graph of the average percentage of TUNEL-positive cells in the border zone area surrounding the infarction for each experimental group. (B) Representative TUNEL-stained cross-sections from a single animal in the Null and HGF experimental groups (original magnification ×200). Apoptotic nuclei are stained dark brown (red arrows) and normal nuclei are stained blue-green (Sham n=10, Null n=9, HGF n=10). *$P<0.01$ versus Null control.

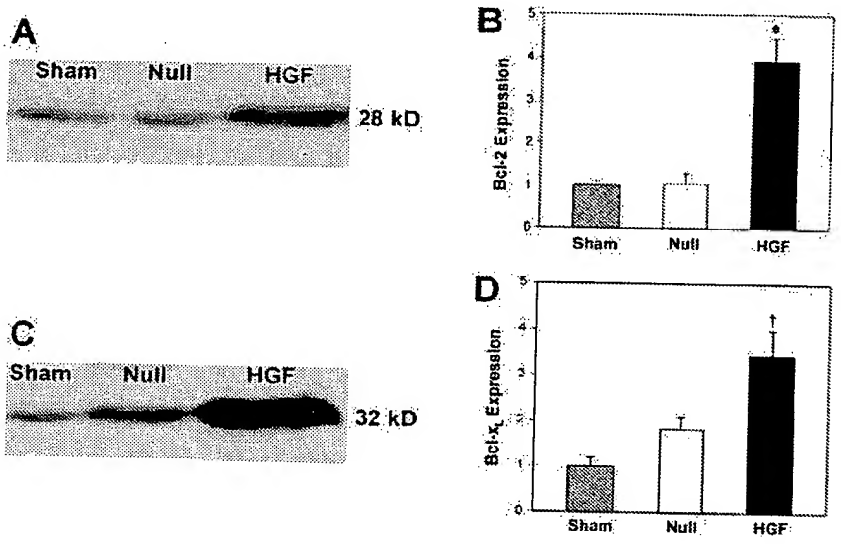

Figure 7. (A) Representative results of immunoblotting for Bcl-2 protein in the border zone area surrounding the infarction at the study end point. (B) Graph of average relative expression of Bcl-2 protein in each experimental group. (C) Representative immunoblotting results for Bcl-x_L expression. (D) Graph of average relative expression of Bcl-x_L protein. All values were normalized to expression levels in the Sham group. *$P<0.01$ versus Null control, †$P<0.001$ versus Null control.

ischemia, and studies have confirmed that late reperfusion after infarction benefits ventricular remodeling and function.[18,19] Angiogenesis may be particularly effective in the early postinfarction period, when significant areas of border-zone myocardium are viable but hypo- and noncontractile because of ischemia. Improved perfusion would, therefore, both reduce ischemic cell loss and improve global cardiac function.

Ventricular remodeling with chamber dilatation and wall thinning is an important component of post-infarction cardiac failure.[20] The remodeling process has been associated with cardiomyocyte death in and around the region of infarct.[21] Hepatocyte Growth Factor has potent anti-cell death effects,[22] and has specifically been shown to inhibit apoptotic cell death,[7] which is known to contribute significantly to postischemic injury in the heart.[8] In our study we observed upregulation of both Bcl-x_L and Bcl-2 with HGF treatment, both of which have anti-apoptotic actions. Interestingly, infarcted Null control animals had an almost 2-fold increase in Bcl-x_L expression compared with Sham controls, although no similar increase in Bcl-2 expression was seen. This may be an adaptive response to blunt some of the apoptotic response following myocardial infarction. We hypothesize that blocking the activation of apoptosis following myocardial injury prevents cell loss and preserves myocardial geometry and function, as we have previously demonstrated in a rabbit model of chronic ischemia.[23] Since ventricular volume has been shown to be a positive predictor of mortality in heart failure,[24,25] the ability of HGF to limit ventricular dilatation is particularly beneficial. In fact, HGF overexpression significantly preserved myocardial function such that Adeno-HGF treated rats with large infarcts did not have significantly different hemodynamic parameters than Sham uninfarcted animals.

The introduction of HGF in a clinical setting may be performed at the time of acute infarction with an open or percutaneous catheter-based method. Direct intramyocardial injection of the adenoviral vector could be easily performed during open or minimally invasive bypass surgery, or during percutaneous interventions. Targeting of the border zone area surrounding the infarct can be accomplished either by direct visualization in the operating room, or by assessment of noncontractile areas in the cardiac catheterization laboratory. We have demonstrated that HGF expression is not necessary at the time of infarction, as adenoviral vectors generally take 2 to 3 days to begin expression. Delivery of the transgene via a viral vector could therefore be done in conjunction with ongoing interventions after an acute infarction.

One limitation of this study is that animals were assessed while viral transgene overexpression was still ongoing. Since adenoviral vectors generally lose expression after 4 to 6 weeks, there may be a substantial decline in beneficial effects once HGF expression is gone. It may be that only early expression of HGF is needed, and that the angiogenic effect confers a lasting benefit via improved long-term perfusion of the borderzone. On the other hand, the anti-apoptotic effects of HGF may be of more importance, and once this is lost the heart may decline into dilated failure. Further studies using long-term endpoints of 12 and 18 weeks are currently in progress to address this issue. If long-term expression is needed for lasting benefit, strategies employing adeno-associated viral vectors will need to be devised.

In conclusion we have demonstrated that Hepatocyte Growth Factor gene transfer following a large myocardial infarction results in preserved cardiac function and geometry, and that this is associated with significant angiogenesis and a reduction in apoptosis. This therapy may be useful as an adjunct or alternative to standard revascularization techniques in patients with ischemic heart failure.

Acknowledgments

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